Award Number: W81XWH-10-1-0814

TITLE: Development of a Small Molecule P2X7R Antagonist as a Treatment for Acute SCI

PRINCIPAL INVESTIGATOR: Steven Goldman, MD, PhD

CONTRACTING ORGANIZATION: University of Rochester
Rochester, NY 14642

REPORT DATE: October 2013

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED *(From - To)*
October 2013	Final	15 Sep 2010 - 14 Sep 2013

4. TITLE AND SUBTITLE
Development of a Small Molecule P2X7R Antagonist as a Treatment for Acute SCI

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0814

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Steven A. Goldman, MD, PhD
Steven goldman@urmc.rochester.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)
University of Rochester
Rochester, NY 14642

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and
Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

We have identified the transcriptional events associated with spinal cord injury (SCI) in FACS-isolated spinal astrocytes and microglia, with a focus on P2X7R-dependent transcription. We did so by establishing fluorescence-activated cell sorting (FACS) protocols by which we extracted distinct populations of spinal astrocytes and glial progenitor cells from both injured spinal cords and their normal controls. We then microarrayed the expressed RNAs of contused spinal cords, as well as from matched uninjured controls, so as to establish the injury-associated, phenotype-specific patterns of gene expression by astrocytes as microglia. These data have allowed us to define the cell-type specific responses of astrocytes and microglia to SCI, as well as the injury-specific paracrine interactions between these phenotypes, and the potential role of P2X7R in those interactions. Using this information, we identified a number of targets for therapeutic intervention. Among these, we found that pleiotrophin, a negative regulator of the receptor tyrosine phosphatase-β/ζ (PTPRZ1) of glial progenitor cells (GPCs), can relieve PTPRZ1's suppression of progenitor cell expansion, and potentiate oligodendrocyte production. In addition, our bioinformatics analysis has identified novel drug combinations with potential therapeutic value, over and above P2X7R inhibitors, that should provide ample opportunities for new, glial-targeted drug development in SCI.

15. SUBJECT TERMS Spinal cord injury, weight drop injury, P2X7 receptors, locomotor function, experimental model, rat, mouse, small molecule experimental treatment, microglia, astrocytes

16. SECURITY CLASSIFICATION OF			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	13	19b. TELEPHONE NUMBER *(include area code)*
U	U	U			

Table of Contents

Development of a Small Molecule P2X7R Antagonist as a Treatment for Acute Spinal Cord Injury
Steve Goldman MD, PhD

Introduction

In year 3 of this project, we continued to focus on the transcriptional events associated with spinal cord injury (SCI) in isolated spinal astrocytes and microglia, concentrating on P2X7R activity-dependent transcription. In year 1, we established the injury-associated expression profiles of sorted adult spinal microglia, which we have assessed this past year with the goal of defining optimal molecular targets for microglial modulation. This analysis validated the innate immune system as a critical initiator of spinal inflammatory injury, and suggested a number of new targets for therapeutic intervention in SCI.

In year 2 we further developed and validated a set of new FACS protocols by which we have isolated distinct populations of spinal astrocytes and glial progenitor cells, from both normal and injured spinal cords. Using these protocols, which are based on Glut1 and GFAP-dependent astrocytic sorting, and both A2B5 and CD140a-dependent isolation of spinal glial progenitors, we prepared RNAs from contused spinal cords as well as from matched uninjured controls, for microarray analyses.

In year 3, we analyzed the resultant phenotype-specific gene expression data, as dual functions of both injury and time after injury, and identified a discrete set of astrocytic and microglial genes dysregulated by SCI, as well as a set of specific ligand-receptor pairs potentially involved in the response of glial cells to microglial activation following injury.

With the acquisition of these data –which necessitated the first-ever FACS isolation of defined glial phenotypes from the injured adult central nervous system - we successfully completed our planned identification of paracrine interactions between spinal astroglia and microglia in response to SCI, and of the role of P2X7 receptor activation in that process. Indeed, in parallel work using uninjured human brain tissue-derived cells, intended to validate the targets identified in our mouse injury screen as of human relevance, we have already identified a potentially important paracrine loop between vascular cells and glial progenitor cells, which may regulate reactive gliosis and glial scar formation, as well as remyelination after injury. Specifically, we found that the endothelial and astroglial cytokine pleiotrophin, which acts as a negative regulator of the receptor tyrosine phosphatase-β/ζ (PTPRZ1) of glial progenitor cells, relieves the tonic suppression of progenitor cell expansion otherwise provided by PTPRZ1, and can thereby mobilize endogenous glial progenitor cell pools. Pleiotrophin has been reported to be upregulated in the setting of injury, and may thus contribute to both reactive gliosis and compensatory remyelination; we continue to assess under what conditions these two very distinct glial fates are instructed, and how that process may be manipulated for therapeutic purposes.

Thus, on the basis of the injury-associated, phenotype specific gene expression data obtained in this study, we have already identified promising targets for therapeutic intervention, over and above the P2X7R-dependent transcripts with which we are primarily focused.

Progress summary

1. We established both single- and multi-color sorting protocols by which microglia, astrocytes, and endothelial cells could be concurrently sorted to purity from the adult murine spinal cord

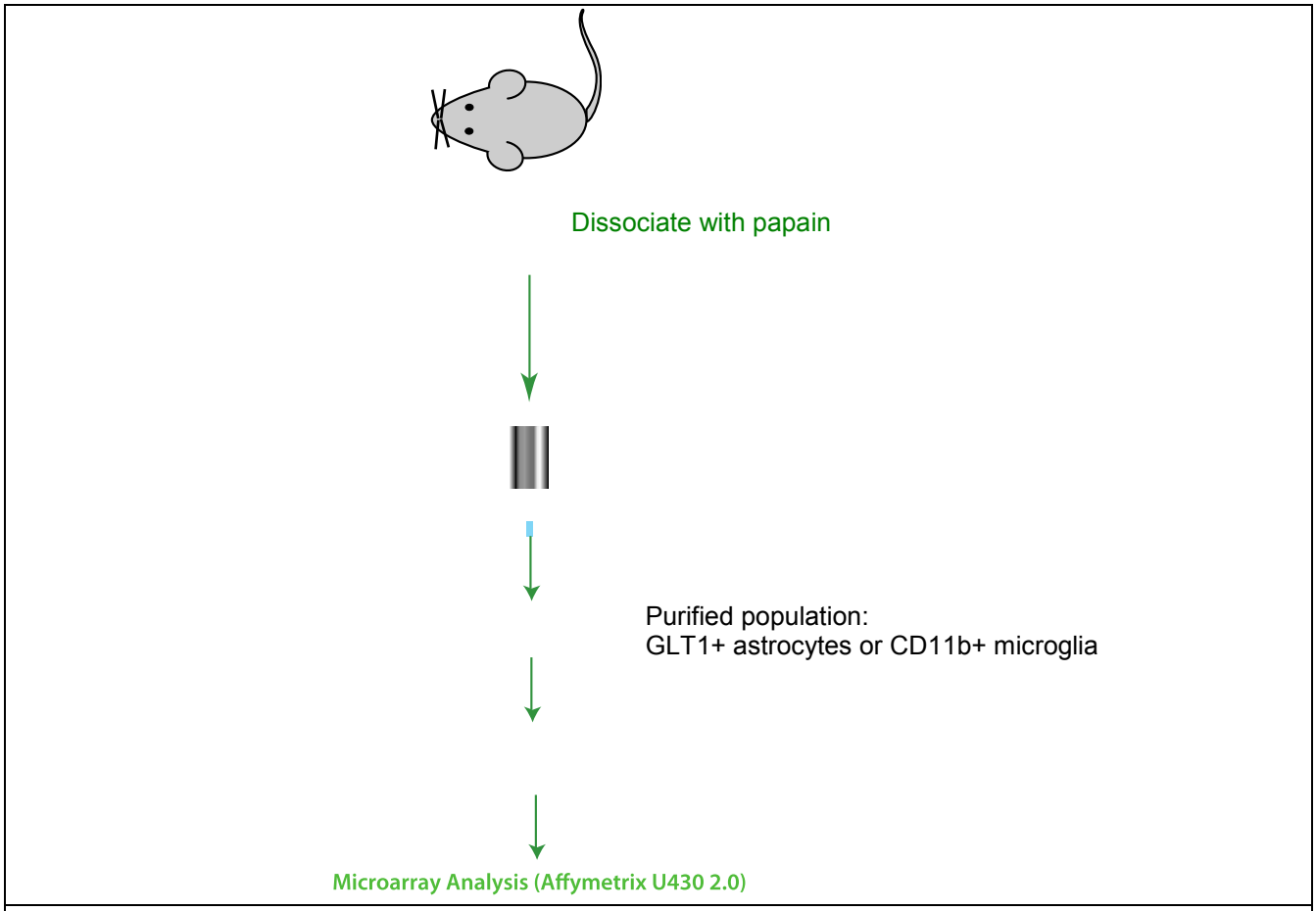

Figure 1 Spinal cords were dissected from 8-week old adult female mice (C57BL6). Control mice: ~1 cm of LSC tissue, with center at ~T10; SCI mice: ~1 cm of LSC tissue, with center at site of injury (at ~T10). The animals were sacrificed at 24 hrs, and spinal cord tissue was dissociated with papain. A Percoll gradient was performed to remove myelin, debris and blood cells. The cells were then immunostained live for GLT1 and CD11b, and flow cytometry with FACS was performed.

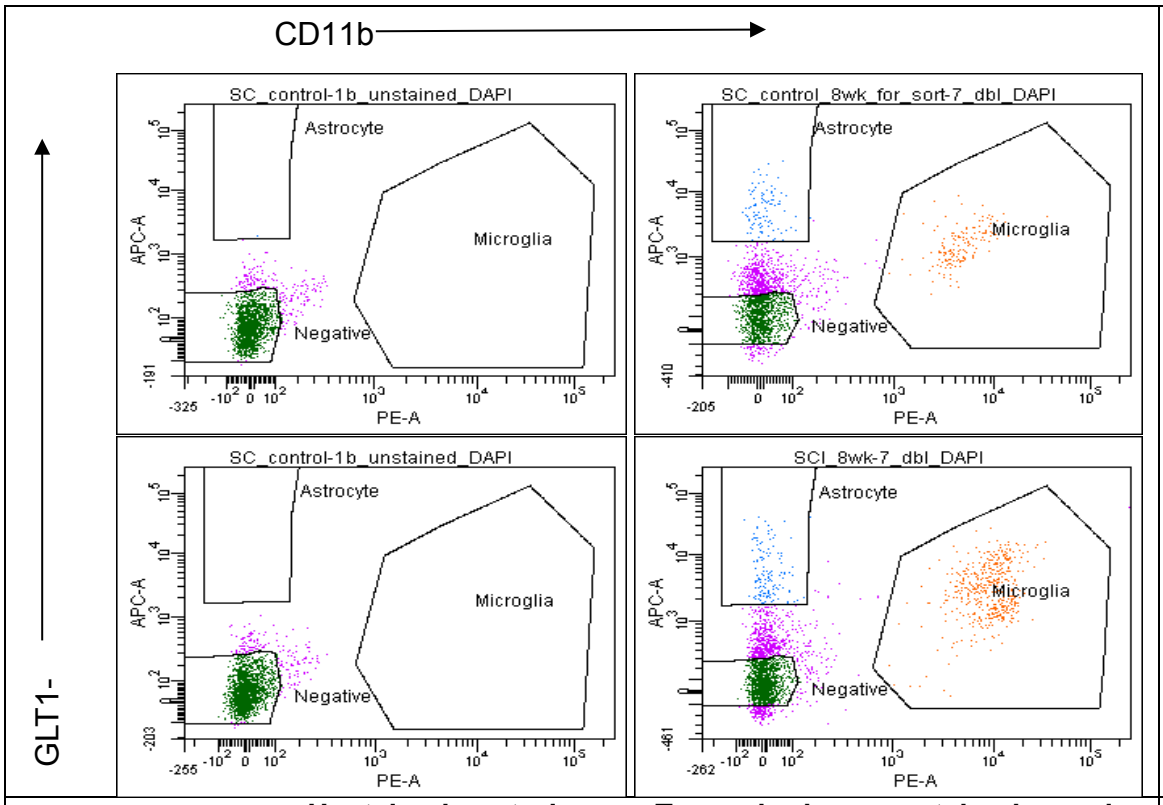

Figure 2 *Isolation and purification of adult murine spinal astrocytes and microglial cells by two-color fluorescence activated cell sorting* Flow cytometric assessment and high-yield enrichment of spinal cord astrocytes (Glt1^{+}) and microglia (CD11b^{+}) from both injured and control mouse spinal cords, 24 hrs post-injury (vs uninjured control).

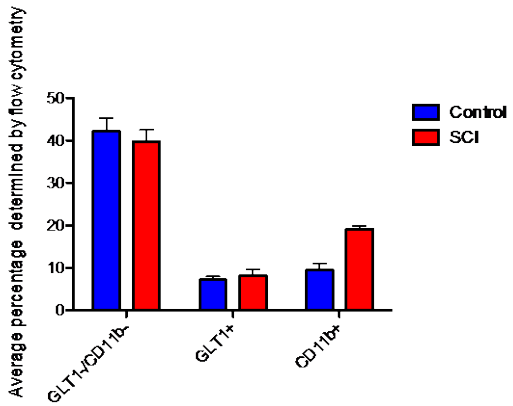

Figure 3 *Prevalence of spinal astroglia and microglia as a function of SCI*

Injured thoracic segments were collected so as to include 2 segments above and below weight drop impact site; samples taken at 24 hrs post-injury. Astrocytes identified and sorted based upon GLT1 expression; microglia defined by CD11b immunoreactivity. N≥4 flow cytometry runs each.

Uninjured	% GLT1-/CD11b-	% GLT1+	% CD11b+
control	42.23	7.15	9.43
SE control	3.02	0.83	1.59
Injured	% GLT1-/CD11b-	% GLT1+	% CD11b+
SCI	39.75	8.08	19.13
SE SCI	2.76	1.58	0.64

2. We profiled the expressed mRNAs of murine spinal astroglia from both spinal cord-injured and uninjured control mice, as a function of time after injury

Mouse age	Type	Sample Info	# cells (events)	RNA conc.	Vol (µl)	tot RNA (ng)	260/280	RIN
			Uninjured controls					
8 wk	wt	GLT1+	82089	4.00	23.8	95.20	1.69	9.7
8 wk	wt	CD11b+	76181	6.47	23.8	153.99	1.83	8.6
8 wk	wt	GLT1-/CD11b-	123714	10.22	23.8	243.24	1.57	8.5
8 wk	wt	GLT1+	110513	2.12	28.8	61.06	2.46	8.8
8 wk	wt	CD11b+	178342	3.30	28.8	95.04	2.33	9.9
8 wk	wt	GLT1-/CD11b-	302191	8.33	28.8	239.90	1.73	9.7
8 wk	wt	GLT1+	91587	0.97	28.8	27.94	-8.52	8.3
8 wk	wt	CD11b+	151494	4.10	28.8	118.08	1.84	9.9
8 wk	wt	GLT1-/CD11b-	505607	4.06	28.8	116.93	2.43	9.7
8 wk	wt	GLT1+	68889	14.25	28.8	410.40	1.5	7.6
8 wk	wt	CD11b+	93733	9.66	28.8	278.21	1.67	7.7
8 wk	wt	GLT1-/CD11b-	478360	1.86	28.8	53.57	1.65	9.2
			Spinal cord injured samples					
8 wk	SCI	GLT1+	84000	6.09	23.8	144.94	1.81	9.7
8 wk	SCI	CD11b+	289000	5.57	23.8	132.57	2.15	10
8 wk	SCI	GLT1-/CD11b-	423722	2.89	23.8	68.78	2.01	9.5
8 wk	SCI	GLT1+	123000	4.81	23.8	114.48	1.76	2.6
8 wk	SCI	CD11b+	267000	5.94	23.8	141.37	2.11	n/a
8 wk	SCI	GLT1-/CD11b-	642000	4.75	23.8	113.05	1.89	n/a
8 wk	SCI	GLT1+	63703	8.37	28.8	241.06	1.62	9
8 wk	SCI	CD11b+	323446	16.59	28.8	477.79	1.78	10
8 wk	SCI	GLT1-/CD11b-	983079	13.02	28.8	374.98	1.86	10
8 wk	SCI	GLT1+	57478	10.19	28.8	293.47	1.67	8.7
8 wk	SCI	CD11b+	193710	9.10	28.8	262.08	1.79	10
8 wk	SCI	GLT1-/CD11b-	543042	6.85	28.8	197.28	1.86	10

Figure 4 *Injured and control spinal cord-derived astrocyte and microglial mRNA samples analyzed* RNA was isolated from all samples and the RNA quality of each sample was confirmed (most RIN values > 8.5). N = 4 sets each of uninjured and injured spinal cords; mRNA samples were profiled on Affymetrix U430 2.0 mouse arrays.

3. We established the injury-associated differentially-expressed mRNA profiles of both microglia and astroglia in the mouse spinal cord

We concurrently sorted both Glt1-defined astroglia and CD11b-defined microglia from injured spinal cords, after developing and validating the techniques for this difficult undertaking. The resultant expression profiling of these sorts has provided us an extraordinarily rich database, from which we have identified those transcripts of each phenotype most dysregulated in response to injury (**Figures 5-6**). From these data, we have defined the dominant signaling pathways, both intracellular and paracrine, affected by acute SCI. Using the Drug Pair Seeker algorithm, we then used these data to predict those pathway-targeted agents that might be used to reverse the injury-associated expression signature, and by that means to rescue the threatened spinal cord.

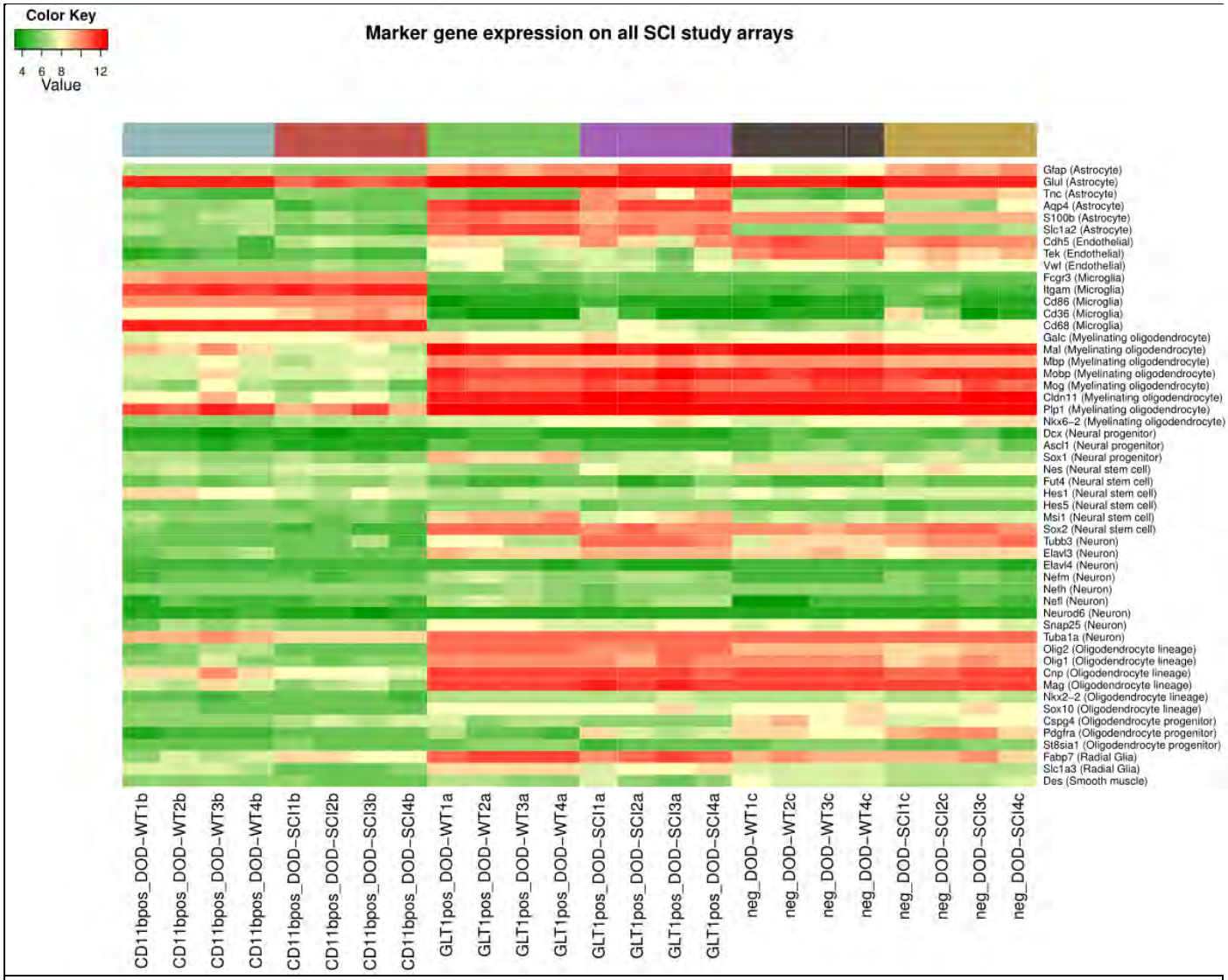

Figure 5 Phenotype-selective gene expression by *mouse* microglia and astrocytes Gene expression by sorted murine spinal cord microglia (CD11b) and astrocytes (GLT1), comparing uninjured and post-SCI samples of each (n=4/group). The intragroup consistency of marker expression validated our sort criteria and selection modalities, and permitted the identification of cell type-specific changes in gene expression as a function of SCI.

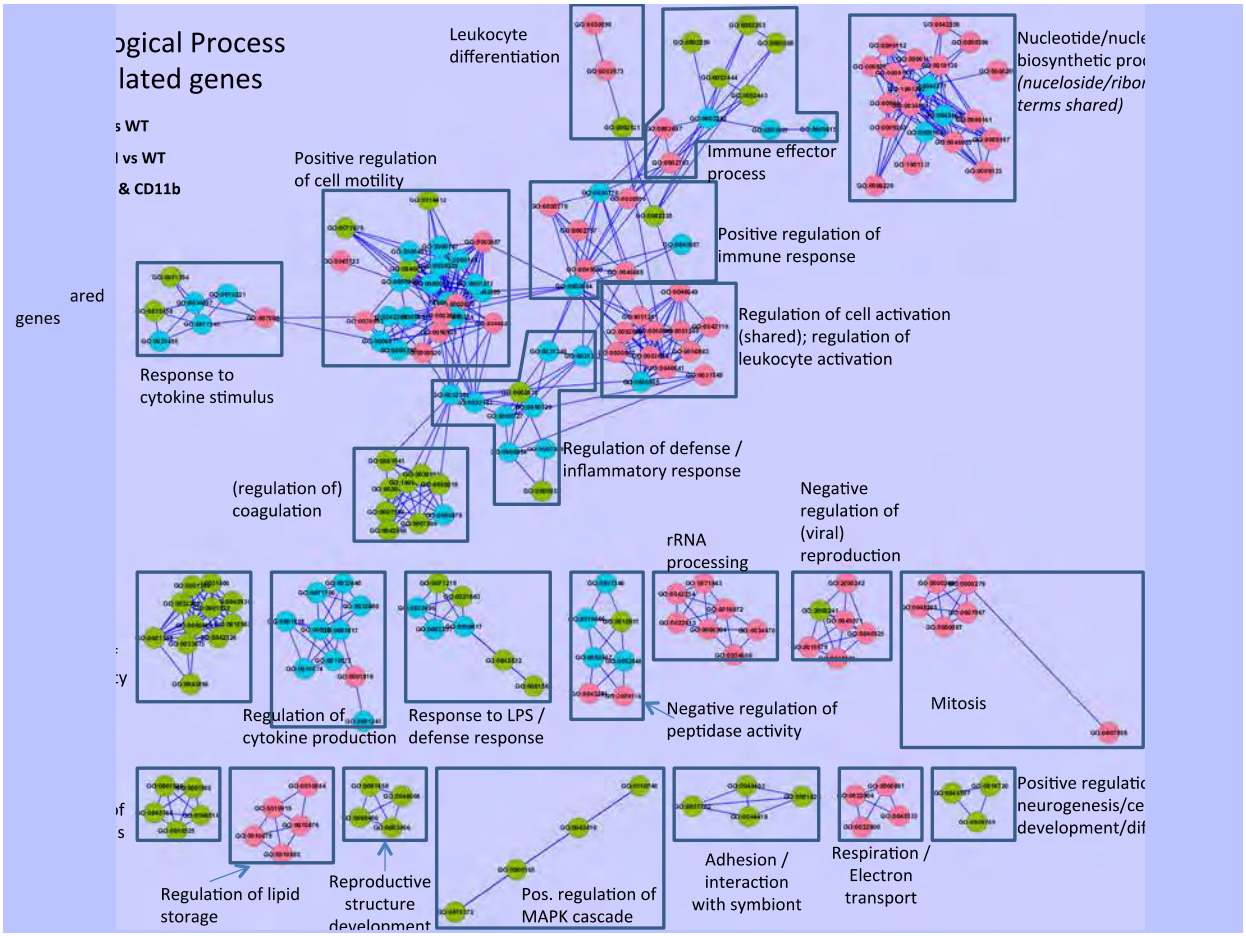

Figure 6 Differentially up-regulated genes in both astrocytes and microglia following injury fall into a set of discrete functional categories, with both phenotype-specific and nonspecific expression.

4. We established the expression profiles of normal adult human microglia, as a cross-species baseline comparator.

In previous studies, we found that the specific genes expressed by homologous human and mouse brain cells can differ substantially, even within otherwise conserved pathways, and with otherwise conserved interactants. As a result, any observations that we make in mouse spinal glia in terms of specific injury-associated transcriptional targets need to be validated in human cells. To that end, we profiled both astrocytes and microglia derived from adult human brain, and analyzed the resultant profiles to capture both commonalities and salient differences between mouse and human microglial gene sets at baseline.

5. We assessed the molecular basis for injury-induced glial progenitor cell mobilization, and found that pleiotrophin-mediated inhibition of PTPRZ1 can permit the sustained expansion of human oligodendrocyte progenitor cells

Our gene expression data have highlighted the importance of the receptor tyrosine phosphatase β/ζ on the homeostatic turnover and differentiated fate of human glial progenitor cells. We first became interested in this receptor since our expression analyses identified its major known ligand, pleiotrophin, as highly expressed by GPCs, providing a possible autocrine loop for the self-maintenance of glial progenitors, the perturbation of which might dictate progenitor recruitment as either reactive glia or myelinogenic oligodendrocytes in the injured CNS. In addition, we noted the expression of pleiotrophin by our arrayed murine spinal microglia, while others have reported its expression by activated endothelial cells; these observations suggest the possibility that injury-associated microglial activation might trigger

pleiotrophin production, and hence mobilize glial progenitor cells from a quiescent state to mitotic expansion and subsequent astrocytic and oligodendrocytic differentiation. We investigated this possibility, first by better defining the signaling pathways by which pleiotrophin-induced suppression of PTPRZ1 triggers mitotic expansion of the glial progenitor pool. We found that PTPRZ1 constitutively promotes the tyrosine dephosphorylation of β-catenin and thus nuclear translocation and β-catenin participation in T cell factor (TCF)-mediated transcription.

Using CD140a/PDGFRα-based fluorescence-activated cell sorting to isolate GPCs from the human fetal brain at gestational ages 16-22 weeks, we then asked if pleiotrophin modulated the expansion of GPCs and, if so, whether this was affected through the serial engagement of PTPRZ1 and β-catenin-dependent signals, including TCF-mediated transcription. We found that lentiviral shRNAi knockdown of PTPRZ1 induced TCF-mediated transcription, and in addition, substantially augmented GSK3β inhibition-induced TCF-reporter luciferase expression. These results suggested dual regulation of β-catenin, and the importance of PTPRZ1 as a tonic brake upon TCF-dependent transcription. Pharmacological inhibition of GSK3β triggered substrate detachment and initiated sphere formation, yet had no effect on either proliferation or net cell number. In contrast, pleiotrophin strongly potentiated the proliferation of CD140a-sorted OPCs, as did PTPRZ1 knockdown, which significantly increased the total number of population doublings exhibited by OPCs before mitotic senescence. These observations indicated that pleiotrophin inhibition of PTPRZ1 mediates the homeostatic self-renewal of OPCs, via the (Wnt-independent) activation of β-catenin/TCF-dependent transcription.

Key Research Accomplishments

1. We established multicolor sorting protocols by which microglia, astrocytes, and endothelial cells may be sorted to purity from the adult murine CNS, and established the numbers and relative proportions of these cells in both the normal and injured adult spinal cord.

2. Using CD11b and Glt1-targeted fluorescence activated cell sorting (FACS), we profiled the expressed mRNAs of murine spinal microglia and astrocytes, respectively, from both spinal cord-injured and uninjured mice. By mRNA expression profiling and bioinformatic analysis of these samples, we defined the phenotype-specific transcriptional responses of mouse spinal microglia and astroglia to SCI, each as a function of time after injury.

3. On the basis of our initial assessments of differential gene expression by glial progenitor cells, we assessed the function of the highly differentially expressed receptor tyrosine phosphatase PTPRZ1. We found that the inhibition of PTPRZ1 by its inhibitory ligand pleiotrophin triggers the sustained expansion of human oligodendrocyte progenitor cells. We concurrently established that the BMP inhibitor noggin could suppress astrocyte lineage commitment, and hence astrocytosis from human GPCs. These observations provide us a strategy and reagents by which to modulate reactive gliosis as well as remyelination in the injured spinal cord.

4. Using our database of SCI-associated differentially expressed astroglial and microglial genes, we defined the dominant signaling pathways, both intracellular and paracrine, affected by acute SCI. In addition, in both cell types we identified those pathways downstream of P2X7R that are differentially modulated by SCI. Using a bioinformatic strategy by which we paired Connectivity mapping to Drug Pair Seeker, we used these data to predict pathway-targeted agents that might be used to reverse the injury-associated expression signature. That analysis yielded a number of drug pair candidates that our algorithms predict capable of reversing significant features of the SCI-associated expression profile; as such, our data would suggest that these drug candidates and combinations thereof should provide substantial benefit to the threatened spinal cord.

5. To verify the effects of P2X7R antagonism on gene expression by *human* astrocytes as well as on their mouse counterparts, we established methods for generating these cells from human induced

pluripotential cells, an advance that should greatly facilitate the modeling of human glial-specific effects of P2X7R-targeted treatment.

Reportable Outcomes

Papers and reviews, Goldman lab, grant year 3 (2013-13)

Oberheim, N., **Goldman, S.A**., Nedergaard, M. Heterogeneity of astrocytic form and function. Methods Mol. Biol. 814:23-45, 2012.

Benraiss, A., Bruel-Jungerman, E., Economides, A., Davidson, B., **Goldman, S.A**. Sustained induction of neuronal recruitment to the adult rat striatum by AAV4-delivered noggin and BDNF. Gene Therapy 19:483-93, 2012.

Iliff, J., Wang, M., Liao, Y., Plogg, B., Peng, W., Benveniste, H., Vates, G. E., Deane, R., **Goldman, S.A.**, Nagelhus, E., Nedergaard, M. A brain-wide gliovascular pathway facilitates the clearance of interstitial solutes including amyloid ß. Science Transl. Med. 4:147ra111, 2012.

McClain, C., Sim, F., **Goldman, S.A.** Pleiotrophin suppression of receptor tyrosine phosphatase β/ζ maintains the self-renewal competence of fetal human oligodendrocyte progenitors. J. Neuroscience 32:15066-15075, 2012.

Goldman, S.A., Nedergaard, M., Windrem, M. Glial progenitor cell-based treatment and modeling of neurological disease. Science 338:491-494, 2012.

Sim, F., **Goldman, S.A**. Gene expression patterns of oligodendrocyte progenitor cells and oligodendroglia. In: Neuroglia, 3d ed., H. Kettenmann and B. Ransom, Eds. Chapter 29, pp. 358-373. Oxford Univ. Press, 2012.

Wang, S., Bates, J., Li, X., Schanz, S., Chandler-Militello, D., Levine, C., Maherali, N., Studer, L., Hochedlinger, K., Windrem, M.S., **Goldman, S.A**. Human iPS cell-derived oligodendrocyte progenitor cells can myelinate and rescue a mouse model of congenital hypomyelination, Cell Stem Cell 12:252-264, 2013.

Goldman, S.A., Ye, R., Chen, Z. Testosterone modulated angiogenesis and neurogenesis in the adult songbird brain. Neuroscience 239:130-148, 2013.

Han, X., Chen, M., Wang, F., Windrem, M., Wang, S., Shanz, S., Xu, Q., Oberheim, N., Bekar, L., Betstadt, S., Silva, A., Takano, T., **Goldman, S.A.,** Nedergaard, M. Human glia potentiate synaptic plasticity in adult mice. Cell Stem Cell 12:342-53, 2013.

Guo, H, Zhao, Z, Yang, Q, Wang, M, Bell, R.D., Wang, S, Chow, N, Davis, T.P., Griffin, J.H., **Goldman, S.A.**, Zlokovic, B.V. An activated Protein C analog stimulates neuronal production by human neural progenitor cells via a PAR1-PAR3-S1PR1-Akt pathway. J. Neuroscience 33:6181-6190, 2013.

Goldman, S.A. White matter from fibroblasts. Nature Biotechnology 31:412-413, 2013.

Benraiss, A., Toner, M., Xu, Q., Bruel-Jungerman, E., Wang, F., Economides, A., Davidson, B., Kageyama, R., Nedergaard, M., **Goldman, S.A.** Sustained mobilization of endogenous neural progenitors delays disease progression in a transgenic model of Huntington's disease, Cell Stem Cell 12:787-99, 2013.

Auvergne, R., Sim, F., Wang, S., Chandler-Militello, D., Burch, J., Al-Fanek, Y., Davis, D., Benraiss, A., Walter, K., Achanta, P., Johnson, M., Quinones-Hinojosa, A., Natesan, S., Ford, H., **Goldman, S.A.** Transcriptional distinctions between normal and glioma-derived glial progenitor cells identify a core set of dysregulated genes. Cell Reports 3:2127-41, 2013.

Goldman, S.A., Osorio, J. Too many progenitors, too little myelin. Nature Neuroscience, 17:483-485, 2014.

Kondo, Y., Windrem, M., Zou, L., Chandler-Militello, D., Schanz, S., Kazarov, A., Gorelik, L., **Goldman, S.A.** JC virus-infected human glial chimeric mice reveal progressive multifocal leukoencephalopathy to be an astrocytic disease. J. Clin. Invest., 2014, *in revision.*

Windrem, M., Schanz, S., Munir, J., Wang, S., **Goldman, S.A.** A competitive advantage by neonatally engrafted human glial progenitors yields mice whose brains are chimeric for human glia. J. Neuroscience, 2014, *in revision*.

Lou, N., Takano, T., Pei, Y., Yu, H., Cook, M., **Goldman, S.A.,** Nedergaard, M. Microglial P2Y12 receptors play a neuroprotective role in ischemic stroke. Nature Medicine, 2014, *in revision*.

Fox, I., **Goldman, S.A.,** Huard, J., Kamp, T., Trucco, M. Pluripotential stem cells as therapy. Science, 2014, *in revision.*

Wang, S., Chandler-Militello, D., Cornwell, A., **Goldman, S.A**. Differential gene expression by spinal astrocytes and microglia in response to acute traumatic cord injury. PLoS One, *submission*.

Conclusions/Future Plans

In this 3-year project, we succeeded in defining the patterns of injury-associated differential gene expression by spinal microglia and astroglia. We used these data to reconstruct the paracrine networks dysregulated by injury, and have, as intended, defined a set of molecular targets whose modulation may permit the attenuation of the spinal inflammatory response. Using this data set, we have predicted a number of molecular targets, both microglial and astrocytic, for potential therapeutic intervention, and have used bioinformatic predictive algorithms to identify drug combinations that may prove competent to reverse enough injury-associated gene expression to have therapeutic value. Furthermore, our bioinformatics analysis of these primary data sets has allowed us to identify injury-associated pathways potentially modulated by P2X7R. These data should provide a strong referential base against which to assess the effects of P2X7R antagonism on both astrocytes and microglia, and by extension on their neuronal neighbors. By this means, we hope to define the contribution of those pathways downstream of P2XR7 to SCI-associated neuropathology, and the potential for bioinformatically-predicted pharmacotherapy to act in concert with P2X7R-targeted therapeutics in relieving SCI. Together, these studies have succeeded in providing us a strong molecular base for IND-enabling studies of P2X7R inhibitors for the treatment of acute SCI, while identifying an additional set of novel targets and discrete agents by which to potentially treat acute SCI.

Note

This project has generated a large corpus of genomic data that should be of great interest to investigators in SCI research, but which are too detailed for this summary progress report. All genomic data, whether in raw, processed or summary form, can be made available to DOD staff at any time upon request. Upon acceptance for publication, we will make all of these data publically available via GEO (the NCBI Gene Expression Omnibus), as well as by the genomics atlas of our laboratory website (www.urmc.rochester.edu/labs/goldman-lab/genomics). We would expect this to occur no later than the fall of 2014.

Scientific Personnel supported by this project:

Sumedha Bhagat, Sr. Technical Associate
Devin Chandler-Militello, Sr. Technical Associate
Steven Goldman, PI
Su Wang, Research Associate Professor

Report of Inventions

Nothing to report